10

INVOLVEMENT OF PRO- AND ANTI-INFLAMMATORY CYTOKINES IN SEPSIS

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The appearance of detectable pro- as well as anti-inflammatory cytokines in the blood stream during sepsis is indicative of their exacerbated production. The interaction of microorganisms and their derived products with host cells rapidly leads to the production of many inflammatory mediators including cytokines. Two major features characterize the production of these factors: cascade and regulatory loops (Figure 1). This means that, once produced, a given cytokine can induce the production of others which can further induce cytokine release or on the contrary down-regulate the upper-stream synthesis. Usually absent from the plasma at homeostasis, many cytokines are produced in such large amount during sepsis that they can be detected in the circulation of the patients.

While we will not focus our review on this aspect, it should be kept in mind that the production of these inflammatory cytokines is an integral part of the processes initiated by the innate immune response to fight infection.

SEPSIS IS ASSOCIATED WITH AN EXACERBATED PRODUCTION OF ANTI-INFLAMMATORY CYTOKINES AND MEDIATORS

Interleukin-1 (IL-1α, IL-1β)

Involvement of IL-1 in Sepsis

The network of inflammatory events is mainly orchestrated by interleukin-1 (IL-1) and tumor necrosis factor (TNF). Injection of IL-1 into animals results in hypotension, increased cardiac output and heart rate, leukopenia, thrombocytopenia, hemorrhage, and pulmonary edema [1]. Cyclooxygenase inhibitors greatly prevent these different effects. IL-1 receptor antagonist (IL-lra), a natural IL-1 inhibitor, reduces mortality from endotoxic shock [2].

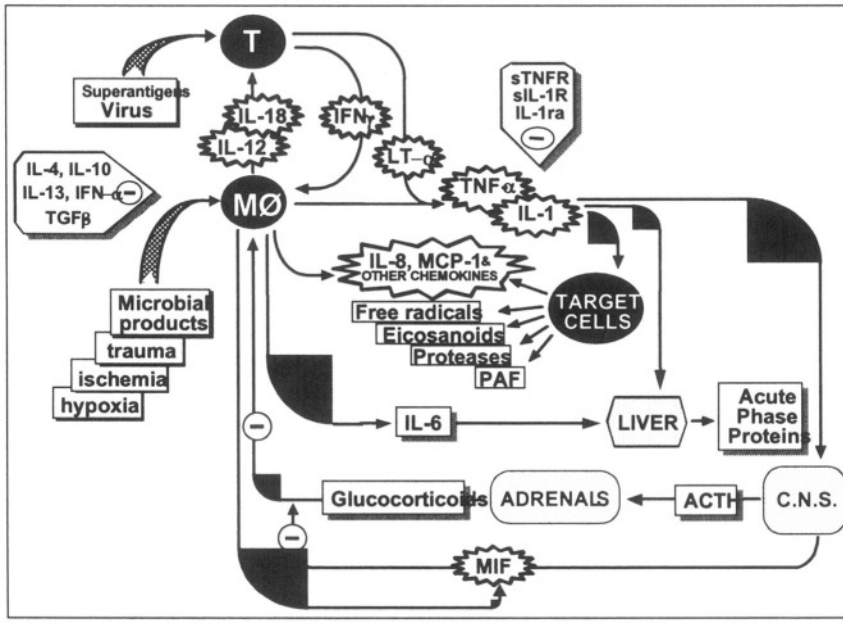

***Figure 1**. Cytokine loops in inflammation and sepsis*

IL-1β converting enzyme (ICE) or caspase-1 is the enzyme required for the maturation of the 30 kDa biologically inactive IL-1β precursor to the mature

17 kDa active form of IL-1β. Survival to a lethal dose of endotoxin reaches 70% among ICE-deficient animals [3], while IL-1β deficient mice are normally sensitive to the lethal effect of LPS [4]. These results reflect that caspase-1 is also involved in the maturation of IL-18.

Detection of IL-1 in Sepsis

IL-1β has been regularly reported in plasma of sepsis patients whereas IL-1α has never been observed when investigated. IL-1β was found in 0 to 90% of septic patients depending on the studies, the nature of the sepsis and on the nature of the technique used to assess its presence. The highest frequency of detectable levels of IL-1β was observed among patients with meningococcal sepsis [5,6] and high levels of IL-1β correlate with the severity of meningococcemia, the presence of shock, high APACHE II scores and rapid fatal outcome [5,6,7,8]. Such correlations were not observed in other sepsis patients [9]. In a few studies, IL-1β survey was performed, and either high levels at admission followed by a decrease, or sustained levels, were reported [6,9,10].

Tumor Necrosis Factor (TNF)

Involvement of TNF in Sepsis

TNF-α toxicity includes hemodynamic instability, fever, diarrhea, metabolic acidosis, capillary leak syndrome, activation of coagulation, late hypoglycemia, induction of a catabolic state, neurotoxicity, cachexia, and renal and hematological disorders, all phenomena associated with sepsis syndrome [11]. In addition, together with IL-1, TNF induces on endothelial cells the expression of adhesion molecules involved in organ infiltration by leukocytes. A lethal effect of TNF was synergistically enhanced by IL-1 [1], interferon (IFN)-γ [12] and lipopolysaccharide (LPS) itself [13]. Anti-TNF treatments have been shown to be highly efficient in protecting animals against endotoxic shock [14] and lethal bacteremia [15]. Such treatments also protected against pulmonary microvascular injury after intestinal ischemia injury which is associated with endotoxin translocation [16]. Studies with mice rendered deficient for TNF or its receptors led to controversial results which reflected the different models - use of D-galactosamine, injection of bacteria, cecal ligation and puncture, injection of high dose LPS - and, as

recently suggested by van der Meer's group in Nijmegen, differences in the bacterial origin of the LPS itself [17].

Detection of TNF in sepsis

In 1986, TNF was the first cytokine to be described in the serum of patients with septicemia [18], and later in patients with meningococcal sepsis [7,19]. While a correlation exists between poor outcome and high levels of measured circulating TNF in the case of meningococcal sepsis [7,19], in other forms of sepsis, some authors did observe such a correlation [10,20], while others did not [9,21]. Different authors have followed up the kinetics of plasma TNF and observed either an increase, a decrease, or sustained levels [9,10,20]. Indeed, as first shown by Baud et al. [21] and confirmed by Pinsky et al. [22], it seems that it is the persistence of detectable TNF rather than its peak level which is associated with the fatal outcome. When addressed, the TNF levels were found to correlate with the severity of illness and APACHE II scores [20,21]. It is worth noting that in intraperitoneal sepsis, on the contrary, high levels of circulating TNF are associated with a good prognosis while low levels correlated with fatal outcome [23,24]. Some authors reported that the TNF levels were higher in Gram-negative than in Gram-positive sepsis although this was not observed in all studies. In meningococcal sepsis, levels of TNF are higher in cerebrospinal fluids than in plasma [25] and not detected in the cerebrospinal fluid (CSF) of patients with non-bacterial meningitis [26]. Injection of LPS in human volunteers and in animal models leads to a plasma peak of TNF at 90 min, and its levels may be up-regulated by administration of ibuprofen [27] or G-CSF [28] and down-regulated by epinephrine [29].

Lymphotoxin-α (LT-α)

Lymphotoxin-α is a rare cytokine which is produced by a limited number of cells, essentially activated T-lymphocytes. It shares with TNF-α the same receptors and thus most of its activities. LT-α should be essentially expected in Gram-positive sepsis since Gram-positive bacteria release various T-cell activators known as superantigens. While the use of neutralizing antibodies could suggest that *Pseudomonas aeruginosa* infusion led to the appearance of TNF-α and LT-α in the circulation of pigs [30], LT-α has never been reported in human Gram negative sepsis [8]. On the contrary, in patients with

streptococcal toxic shock syndrome, circulating LT-α was found to parallel the levels of the circulating superantigen [31].

Interleukin-2 (IL-2)

IL-2 is another cytokine which reflects T cell activation. While rarely reported in human sepsis [8,22], IL-2 was found in the circulation within two hours following injection of bacterial superantigens in mice [32] and baboons [33].

Interleukin-15 (IL-15)

IL-15 shares many functions with IL-2. The specific IL-15 receptor α-chain is associated with the IL-2 receptor β and γ-chains. While IL-2 is mainly produced by T lymphocytes, IL-15 is produced by endothelial cells and by monocytes/macrophages in response to exogenous stimuli such as bacteria and LPS. Importantly, it is expressed on the cell surface as an active molecule [34]. *In vivo,* it is induced by IL-12 [35]. In concert with other monokines (e.g. IL-12), IL-15 stimulates IFN-γ production by natural killer (NK) cells and is involved in the LPS-induced general Shwartzman reaction [36]. However, inhibition by specific antibodies of endogenous IL-15 production during *in vitro* LPS activation of murine macrophages further amplified TNF-α production [37]. The role of IL-15 during sepsis remains to be fully characterized while its presence has been reported in the plasma of septic patients [38].

Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF)

Involvement of LIF and OSM in Sepsis

LIF, CNTF, and OSM belong to the IL-6 superfamily, sharing the gp130 chain of the receptor. However, while IL-6 and IL-11 possess certain anti-inflammatory properties (see below), LIF and OSM can be considered as pro-inflammatory cytokines. Indeed, LIF is involved in the pathogenesis of inflammation and sepsis syndrome [39]. Induced by LPS and TNF, LIF can induce the release of other cytokines including IL-1, IL-6, and IL-8 by

various cell types. Passive immunization against LIF in mice challenged with intraperitoneal administration of endotoxin protected them from the lethal effects and blocked increases in serum levels of IL-1 and IL-6 [40]. Subcutaneous injection of OSM in mice caused an acute inflammatory reaction [41]. OSM favored PMN adhesion to endothelial cells and transmigration via its capacity to enhance the expression of P- and E-selectin, intercellular adhesion molecule (ICAM)-l, and vascular cell adhesion molecule (VCAM)-l. Furthermore, OSM induces the release of IL-6 and ENA78 (an α-chemokine) but not that of IL-8.

Detection of LIF, CNTF, and OSM in Sepsis

First reported in 1992, detectable levels of LIF were occasionally found in plasma of 9 to 40% septic patients [39,42,43]. Levels of circulating LIF correlate with shock, temperature, creatinine and IL-6 [42]. The correlation of LIF with IL-6 has been confirmed in a baboon model of sepsis [44]. Levels of plasma CNTF and OSM are elevated in 60% and 100% septic patients, respectively [43].

Interleukin-8 (IL-8) and Chemokines

Involvement of Chemokines in Sepsis

Sepsis and SIRS are often associated with organ dysfunction that reflects the inflammatory process occurring in the tissues. One of the major features of this phenomenon is the recruitment of inflammatory leukocytes. It implies the adherence of circulating cells to the endothelium and their margination towards the tissues in response to the locally produced chemokines. Chemokines represent a family of more than 40 members. These chemokines contribute to the inflammatory cell infiltrate that participates in the disruption of tissue integrity. For example, neutralization of IL-8 profoundly inhibited neutrophil recruitment in an endotoxin-induced rabbit model of pleurisy, indicating that IL-8 is a major chemotactic factor in this model of acute inflammation [45]. During sepsis a great amount of IL-8 is detectable within the blood compartment, not only as a free cytokine [46] but also as a cell-associated form [47]. This first encounter of neutrophils with IL-8 leads to their desensitization to further signals delivered locally by IL-8. Thus, the presence of IL-8 in the vascular space may well be a mechanism that limits neutrophil accumulation at extracellular sites as illustrated by the defect in

neutrophil migration capacity during sepsis or endotoxemia [48,49]. Similarly, while monocyte-chemoattractant protein-1 (MCP-1) contributes to the recruitment of inflammatory macrophages within the tissues, neutralization of MCP-1 by specific antibodies before LPS administration resulted in a striking increase in mortality and the injection of MCP-1 was protective [50]. In contrast, mice rendered deficient for the receptor of MCP-1 (CCR4-/-) which also binds macrophage inflammatory protein (MIP)-1α, regulated on activation, normal T-cell-expressed and secreted (RANTES), macrophage-derived chemokine (MDC) and thymus- and activation-regulated chemokine (TARC), exhibited significantly decreased mortality on administration of LPS [51]. These controversial results illustrate the influence of the experimental models. Furthermore, one should keep in mind that the recruitment of leukocytes by chemokines is a prerequisite to address the infectious process as elegantly shown by the deleterious effect of blocking MDC in the cecal ligation and puncture model of peritonitis in mice [52].

Detection of Chemokines in Sepsis

As first reported in 1992, a great amount of IL-8 is detectable within the blood compartment during sepsis [53,54] and in broncho-alveolar lavage (BAL) and edema fluid of acute respiratory distress syndrome (ARDS)-associated with sepsis [55]. In this study, patients with high levels of IL-8 in BAL had a high mortality rate. Similarly, high levels of plasma IL-8 correlate with the occurrence of shock [56], with the presence of infectious multiple organ failure (MOF) [46] and with poor outcome [46,53,54]. No difference in IL-8 plasma levels were found between Gram-negative and Gram positive infection [54] while in bacteremic pneumonia the type of pathogen influenced the measurable levels of IL-8 [57]. IL-8 levels also correlate with various markers including IL-6 [5,46,54], C3a, α1-anti-trypsin, lactate [54], IL-10, IL-lra and soluble TNF receptors (sTNFR) [5]. Correlation with plasma TNF led to controversial results [5,58]. More interestingly, local levels of IL-8 often correlate with the number of recruited neutrophils [55] and plasma levels are associated with granulocyte activation as evidenced by massive release of elastase detectable in the circulation of bacteremic baboons [59] and by correlation between elastase and IL-8 in human sepsis [56].

In addition to IL-8, increased levels of various chemokines have been found in plasma of septic patients or following LPS injection in human volunteers. This is the case for MCP-1 and MCP-2 [60], MIP-1α and MIP-1β

[61] and IFN-γ-inducible protein (IP-10) [62], MCP-1 levels being higher in patients with the more severe forms of sepsis (i.e., those with shock or a lethal outcome). In a preliminary study, we found that plasma levels of RANTES were inversely correlated with APACHE II score, and lower levels of this chemokine were found in non-surviving sepsis patients (J-M. Cavaillon and D. Payen, unpublished observation).

Interleukin-12 (IL-12)

Involvement of IL-12 in Sepsis

IL-12 is a heterodimeric cytokine of p40 and p35 subunits. The measurement of p70 heterodimer is correlated with IL-12 bioactivity. IL-12 is a potent inducer of IFN-γ. Its injection in chimpanzees induces an increase in plasma concentrations of IFN-γ as well as IL-15, IL-18, α- and β-chemokines and anti-inflammatory mediators [35]. Among the adverse effects of IL-12, hepato- and splenomegaly, leukopenia, anemia, and myelodepression have been reported [63]. These phenomena were largely IFN-γ-dependent since they were not reported to occur in IFN-γ receptor deficient mice. Hepatomegaly is associated with infiltration of activated macrophages and NK cells, and single-cell necrosis. In contrast, pulmonary edema and interstitial macrophage infiltration generated by IL-12 injection were shown to be IFN-γ-independent. In a Mycobacterium bovis Bacille Calmette-Guerin (BCG)-primed model of LPS-induced shock and lethality, anti-IL-12 antibodies were associated with decreased IFN-γ and were shown to protect mice if injected before endotoxin [64]. In contrast, in a cecal ligation and puncture model, IL-12 neutralization was deleterious [65]. The later observation is in agreement with other reports which demonstrated the beneficial effects of IL-12 in the infectious process [66]

Detection of IL-12 in Sepsis

Bioactive IL-12 was detected in mouse serum at 2 to 4 h after LPS injection [67] and in baboons, surprisingly, higher levels of IL-12 were detectable in plasma of animals injected with sublethal doses of *E. coli* than in animals challenged with lethal doses [68]. In humans, an intravenous bolus injection of *E. coli* LPS in volunteers did not lead to changes in the plasma levels of IL-12 [69] and IL-12 could not be measured in most septic patients [70].

While higher levels of IL-12p40 were found in patients with severe melioidosis (infection with *Burkholderia pseudomallei*) than in healthy controls, IL-12p70, not detectable in controls, was only found in 10% of the patients [38].

Interleukin-18 (IL-18)

Involvement of IL-18 in Sepsis

IL-18 is structurally related to the IL-1 family and its maturation is under the control of caspase-1. Produced by activated macrophages and Kupffer cells, IL-18 is a potent inducer of IFN-γ [71]. While IL-18 promotes resolution of bacterial infection in mice [72], it accounts for both TNF-α and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in this model [73]. Neutralization of IL-18 protects mice against lethal *E. coli* and S. *typhimurium* endotoxemia [74] and IL-18 deficient mice showed decreased sensitivity towards LPS-induced shock [75], although this might depend upon the model since *Propionibacterium acnes*-primed IL-18-/- mice were highly susceptible to LPS [76]. It is worth noting that, in contrast, IL-18-/- mice and normal mice were similarly responsive to bacterial superantigen [75].

Detection of IL-18 in Sepsis

Plasma IL-18 is found in healthy controls and its level was enhanced in patients with melioidosis [38]; levels were higher in bacteremic patients and correlated with APACHE II score, and there was a weak correlation with IFNγ levels ($r = 0.48$).

Interferon-γ (IFN-γ)

Involvement of IFN-γ in Sepsis

IFN-γ is an efficient amplification cytokine produced by T-lymphocytes in response either to IL-12 and/or IL-18 produced by monocytes/macrophages activated by microbial products or by superantigens or viruses. Its synergy with the detrimental activities of LPS has been clearly established: IFN-γ

enhanced LPS-induced circulating TNF-α as well as LPS- and TNF-induced mortality [12,77] and anti-IFN-γ antibodies protected against LPS- and *E. coli*-induced mortality [77,78]. As a consequence, a clinically silent viral infection may induce hypersensitivity to Gram-negative bacterial endotoxin through T cell activation and subsequent IFN-γ production, leading to a hyperproduction of TNF-α [79]. Mice lacking IFN-γ receptor have been shown to be resistant to LPS challenge after priming with BCG [80] or treatment with D-galactosamine [81]. A mouse model of endotoxemia revealed that IFN-γ was not involved in pulmonary edema [82]. Side-effects of IFN-γ include tachychardia, myalgia, malaise, leukopenia, and weakness.

Detection of IFN-γ in Sepsis

The study of circulating IFN-γ in human sepsis led to contradictory results. While in sepsis and purpura fulminans, IFN-γ was found in patients with the most severe disease [7], no correlation was reported with outcome in other studies on sepsis and septic shock [10,22]. No detectable IFN-γ was reported in meningococcal septic shock [8] and in human volunteers receiving systemic endotoxin [69]. IFN-γ was recently detected in the plasma in 71% of patients with melioidosis [38]. In a baboon septic shock model, IFN-γ levels were threefold higher in lethally challenged animals than in those receiving sublethal doses [68].

Interleukin-16 (IL-16) and IL-17

IL-16 and IL-17 are recently described cytokines with ill-defined physiologic properties. Both were discovered as T-cell products, and IL-16 can also be produced by eosinophils, mast cells, and epithelial cells. It is worth mentioning that both can stimulate the production of pro-inflammatory cytokines. IL-16 induces the secretion of IL-1β, IL-6, IL-15 and TNF-α [83] and IL-17 up-regulates the expression of IL-1β, TNF-α, IL-6, IL-12, PGE2 as well as IL-lra and IL-10 [84]. However, their involvement during sepsis has not been addressed so far.

Colony stimulating factors (CSF)

Involvement of CSFs in Sepsis

In addition to their well known action on hematopoiesis, CSFs favor the anti-infectious process and may reduce the natural apoptosis of neutrophils. However, among these cytokines, IL-3, previously called 'multi-CSF', and granulocyte-macrophage CSF (GM-CSF) can amplify the production of IL-1 and TNF-α, and thus behave as pro-inflammatory cytokines. The deleterious effect of GM-CSF is exemplified by the response of GM-CSF-deficient mice to endotoxin: following LPS injection, hypothermia, loss in body weight, levels of circulating IFN-γ, IL-1α, and IL-6 were markedly reduced as compared to normal mice. Furthermore, the survival to one LD100 of LPS was 42% among GM-CSF-/- mice [85].

In contrast, numerous studies have reported that granulocyte-CSF (G-CSF) possesses many beneficial properties. It has been demonstrated that G-CSF reduces endotoxemia and improves survival during *E. coli* pneumonia [86], reduces bacterial translocation due to burn wound sepsis [87], and enhances the phagocytic function of neutrophils in septic animals [88]. Furthermore, in combination with antibiotics, G-CSF can prevent severe infectious complication in a peritonitis model [89] and in combination with IL-11 prevents the occurrence of lethality to a bacterial challenge in neutropenic animals [90].

Detection of CSF in Sepsis

In humans, M-CSF is present at homeostasis in the circulation and its level is increased in patients with sepsis and higher in patients with sepsis-associated hemophagocytosis [91]. G-CSF is also increased in sepsis and reaches higher levels in severe sepsis as compared to sepsis or bacteremia [92]. Enhanced levels of circulating G-CSF have been particularly associated with infection and sepsis in neonates [93]. In meningococcemia, plasma GM-CSF concentrations were briefly present in subjects with life-threatening septic shock and were strongly associated with fulminant disease [92]. GM-CSF was also markedly elevated in septic preterm infants [94].

Fas ligand (FasL)

Fas (CD95) is a member of the TNF receptor superfamily which contains a cytoplasmic death domain. Its ligation with its ligand (FasL) results in the induction of apoptosis. FasL exists as a membrane form or a soluble molecule. While sepsis is associated with a delayed apoptosis of neutrophils, an increased apoptosis in hematopoietic tissues such as thymus, Peyer's patch, spleen and bone marrow has been regularly observed. Using FasL deficient mice, it was established that the sepsis-associated apoptosis of lymphoid cells was a FasL-dependent process [95]. mRNA for Fas and FasL were highly up-regulated in BAL cells during the acute phase of human ARDS following sepsis [96] Enhanced levels of soluble FasL were measured in the BAL of these patients while soluble FasL was absent from BAL of healthy controls.

Macrophage Migration Inhibitory Factor (MIF)

MIF was first discovered in 1966 as a T-cell product released during delayed-type hypersensitivity [97] and rediscovered in 1993 as a pituitary-derived cytokine that potentiates lethal endotoxemia [98]. MIF is now recognized as a macrophage product [99] induced by the action of glucocorticoids [100]. MIF is expressed constitutively in many tissues including lung, liver, kidney, spleen, adrenal gland, and skin. MIF exists as a preformed cytokine which is rapidly released following LPS injection [101]. Bernhagen et al. [98] reported that injection of MIF together with one LD40 of LPS greatly potentiated lethality and that anti-MIF antibodies fully protected against one LD50 of LPS. Accordingly, MIF-deficient mice were more resistant to LPS induced lethality [102]. This phenomenon was associated with a reduced level of circulating TNF, an enhanced level of nitric oxide (NO) and no effect on IL-6, IL-10 and IL-12 levels. Anti-MIF antibodies also protected mice from lethal experimental peritonitis, even when treatment was started up to 8h after induction of peritonitis. [103]. While MIF is present in the plasma of healthy controls, its levels are significantly enhanced in septic patients [103].

High Mobility Group-1 (HMG-1) Protein

HMG-1 is a highly conserved nuclear protein that binds cruciform DNA. It exists as a membrane form and as an extracellular form which interacts with plasminogen and tissue type plasminogen activator (t-PA). It is produced by macrophages in response to LPS and by pituitary cell stimulated by IL-1 or TNF [104]. HMG-1 was reported to be a late mediator involved in endotoxin lethality in mice. HMG-1 has been found in plasma of septic patients, with significantly higher levels in non-survivors than in survivors [104].

Cellular Signaling Induced by Pro-inflammatory Cytokines

The Nuclear Factor-kappa B (NF-κB) and the Mitogen-activated Protein Kinase (MAPK) Pathways

During the inflammatory processes the inducible transcription factor NF-κB plays a major role in the intracellular signaling. Indeed, this is one of the main nuclear factors that regulates the transcription of numerous genes including cytokines, especially pro-inflammatory cytokines such as TNF-α, IL-1α, IL-6 and IL-8, cytokine receptors, acute phase proteins and leukocyte adhesion molecules. These components are important for the recruitment of circulating cells towards the inflammatory focus [105,106]. The NF-κB family is composed of various members, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel, which can form homo- and heterodimers [107]. Numerous studies have shown that the transactivator form of NF-κB is the p65 unit while the p50 unit showed no or minimal activation capacities [108,109,110]. The transactivator form of NF-κB is the p65p50 heterodimer in mammalian cells, although some reports show transactivatory activities of p50p50 in cell-free *in vitro* transcription systems [111,112] or in yeast [113]. Fujita et al. [112] found that p50p50 could behave as a gene activator when complexed to the Bcl-3 protein, but another report shows that Bcl-3 facilitates the NF-κB transactivation by removing the inhibitory p50p50 from the κB-sites [114]. NF-κB is regulated by a cytoplasmic inhibitor: IκB. This protein is also a member of a large family that includes IκBα, IκBβ, IκBγ, IκBε and Bcl-3. All possess multiple regions of homology known as the ankyrin-repeat motifs, also present in the precursors of p50 and p52 (p105 and p100 respectively) which also behave as NF-κB inhibitors.

Among cytokines, TNF and IL-1 are potent activators of NF-κB, but this transcription factor is also inducible by other extracellular signals such as

reactive oxygen species and complement fragments. Furthermore, endotoxin is a potent activator of **NF-κB.** During the past few years, many insights have been reached about the LPS signaling. These include the characterization of Toll-like receptor 4 (TLR4), the co-receptor of CD14, responsible of the signal transduction [115] and that of the MD-2 molecule which is associated to TLR4 at the cell surface [116]. In unstimulated cells, **NF-κB** is retained in the cytoplasm by **IκB** as an inactive complex. As shown in Figure 2, the binding of TNF, IL-1 or LPS to their receptors recruits adaptor molecules which leads to the activation of an **NF-κB-inducing** kinase (NIK) [117]. NIK seems to be the convergent point of the TNF and IL-1-mediated **NF-κB** activation, since mutant forms of NIK block the signaling from the receptors of both cytokines [117]. The final step of the kinase cascade leads to the activation of protein kinases that phosphorylate **IκB.** These **IκB** kinases (IKK) are associated to a high-molecular weight cytoplasmic complex [118]. In addition to NIK, MEKK-1 (a kinase implicated in the c-jun N-terminal kinase [JNK] pathway of MAPK) has been shown to phosphorylate and activate **IKKα** and **β [119].** Recently, a new component of the IL-1RI pathway and a new intermediate in the signal transduction pathway of IL-1 and Toll have been characterized: the first molecule, called Tollip, allows the death domain of MyD88 and IL-1-receptor associated kinase (IRAK) to interact [120]; the second molecule, an adapter protein named evolutionary conserved signaling intermediate in Toll pathway (ECSIT), makes the link between TNF-receptor-associated factor (TRAF)-6 and MEKK-1 [121]. In addition, the characterization of ECSIT also supports the existence of a MEKK-1 -mediated activation of **NF-κB.** After the activation of the IKK, **IκB** is phosphorylated on serines 32 and 36, leading to its subsequent ubiquitination and its degradation by the 26S proteasome pathway. Finally, after the degradation of **IκB,** the **NF-κB** dimer can translocate into the nucleus, bind to DNA and activate the transcription of target genes.

The MAPK cascades are another intracellular signaling pathway activated during the inflammatory process and they also lead to the activation of numerous transcription factors. Three MAPK cascades have been described to date, the extracellular signal-regulated kinases (ERK), the JNK/stress-activated protein kinase (SAPK) and the p38 pathways. The activation of extracellular signal-related kinase (ERK)-1 and -2, also known as p44 and p42, is triggered by mitogens and growth factors, while the two other cascades are activated by IL-1, TNF, LPS and cell stress [122,123]. c-jun is a component of the activator protein (AP)-1 transcription factor and the JNK cascade leads to its phosphorylation and an enhancement of its capacity to activate transcription.

The p38 kinase is implicated in the activation of various transcription factors and some evidence indicates that it can play a role in the activation of **NF-κB.** Indeed, it has been shown that the specific inhibitor of p38 (SB203580) prevented the expression of a reporter gene under the control of **NF-κB** [124]. However, this was not due to an inhibition of the binding of **NF-κB** to DNA. Thus, p38 does not seem to regulate **IκB** phosphorylation, but it most probably modulates the transactivation capacity of **NF-κB** *via* MAPK activated protein kinases (MAPKAP) that in turn phosphorylate the p65 subunit. TNF and IL-1 contribute to the activation of JNK and p38 MAPK.

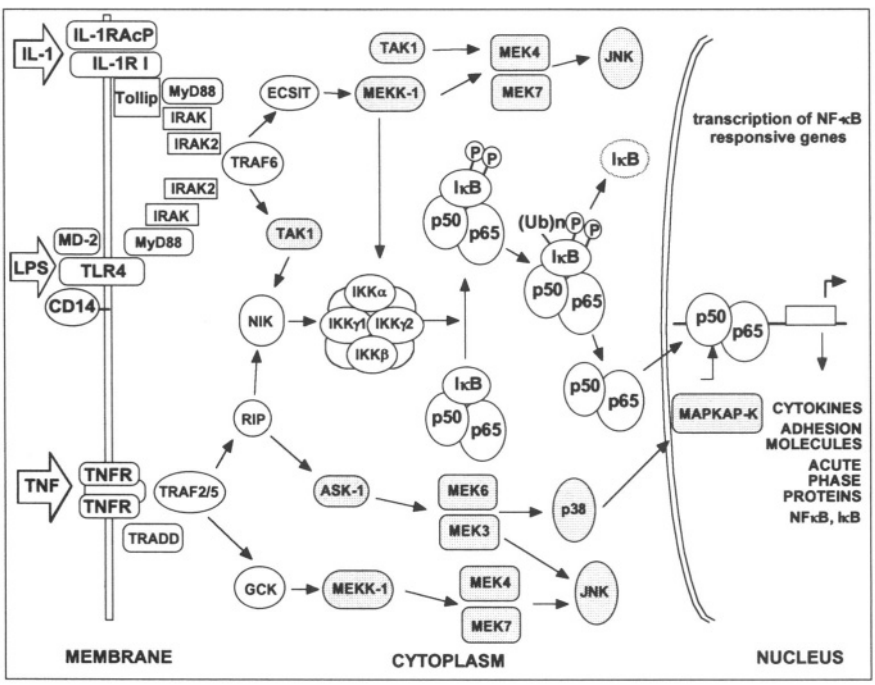

Figure 2. *Cascade of NF-κB activation induced by LPS, TNF and IL-1. The binding of TNF to its receptors activates the NF-κB-inducing kinase (NIK) via its interaction with adaptor molecules: TNF receptor-associated death domain protein (TRADD), receptor interacting protein (RIP) and TNF receptor-associated factor-2 and 5 (TRAF-2/5). LPS signaling requires CD14, the Toll-like receptor 4 (TLR4) and the MD-2 surface protein. IL-1 signaling needs IL-1 receptor accessory protein (IL-1RAcP), the co-receptor of IL-1RI, MyD88 a death domain-containing protein, IL-1 receptor associated kinases (IRAK) that activate NIK via TRAF6 and TAK-1. The evolutionary conserved signaling intermediate in Toll (ECSIT) pathway is the intermediate between TRAF6 and MEKK-1 (a MAPK kinase kinase). p38 another MAPK also contributes to NF-κB activation (NB. the MAPK are represented in gray).*

For TNF, it has been shown that TNF receptor-associated death domain (TRADD), TRAF2 and receptor interacting protein (RIP) are implicated in the signaling leading to JNK and p38 activation [125,126]. Furthermore, another kinase, the germinal center kinase (GCK), has been shown to interact with TRAF2 and MEKK-1 and thus could be the link between the events taking place at the receptor level and the MAPK kinase kinase (MAPKKK) [126]. For IL-1, MyD88 and IRAK are also needed for the signaling. IRAK-deficient mice showed reduced IL-1-mediated JNK and p38 activation [127]. Similarly, overexpression of MyD88 induced the activation of both JNK and NF-κB while mutant forms of MyD88 inhibited their activation [128].

NF-κB in Sepsis: A New Target for Therapy ?

NF-κB has been studied in various *in vitro* and *in vivo* models of sepsis, yet less is known about the status of this transcription factor in humans. Enhanced NF-κB activation has been reported in alveolar macrophages of patients with ARDS [129] and in the lungs and the liver of mice after experimental peritonitis [130,131]. Similarly, after hemorrhage or LPS, NF-κB was activated in lung neutrophils while it was not in circulating neutrophils [132]. The measurement of inflammatory mediators (cytokines, iNOS) also suggests that the consequence of systemic inflammation may differ in the blood and the other tissues and favors the concept of a compartmentalization of inflammation. Indeed, iNOS activity was found to be restricted to the nidus of infection in patients undergoing septic shock after cellulitis [133]. Similarly, after chest trauma, significantly higher levels of IL-1β and IL-8 were found in BAL fluid. In contrast, anti-inflammatory mediators (sTNFRI and II, IL-lra) were present both locally and systemically [134]. Furthermore, an exacerbated production of cytokines has often been demonstrated in non-hematopoietic compartments [135,136], in contrast with the hyporeactivity of circulating cells which will be discussed later. Indeed, the analysis of NF-κB performed with cells derived from the tissues stands in contrast with the experiments performed with peripheral blood mononuclear cells (PBMC). The first reported analysis in septic patients, showed a higher *ex vivo* nuclear expression of NF-κB in the PBMC of the non-survivors [137]. We performed a similar study and confirmed that total NF-κB content is higher in the nucleus of PBMC from non-survivors as compared to survivors of severe sepsis. However, we found that the nuclear p65p50, the active form of NF-κB, was significantly reduced in all patients with severe sepsis as compared to controls and demonstrated that in the non-survivors, NF-κB was mostly composed of the inactive form p50p50 [138].

This down regulation of NF-κB in circulating mononuclear cells was also found when the systemic inflammation was not of infectious origin (major trauma).

Because of its fundamental role in acute inflammation, NF-κB has been chosen by several investigators as a target for the treatment of sepsis. Inhibitors of NF-κB, such as dithiocarbamate (PDTC) or N-acetyl-leucinyl-leucinyl-norleucinal (a potent inhibitor of the proteasome pathway) were tested in animal models of endotoxin shock, encouraged by the inhibiting effect of dithiocarbamate on human immunodeficiency virus (HIV) progression in patients [139]. Treatment with these inhibitors of NF-κB decreased NO synthase (NOS) expression within the tissues [140] and TNF and IL-6 levels in the serum [141], and reduced microvascular injury and disseminated intravascular coagulation (DIC) [142,143]. The limit of these studies is that in all but one [143], the inhibitors had to be administered before the LPS challenge in order to be effective. Furthermore, at high doses, PDTC is toxic and has non-specific effects: it can activate AP-1 another transcription factor that induces pro-inflammatory cytokines. Finally, even if NF-κB is the major transcription factor involved in the pro-inflammatory cascade, its blockade may not be sufficient as other transcription factors, such as AP-1, NF-IL6 or cAMP responsive element binding protein (CREB) can also take part in the induction of inflammatory mediators. Our observation of a dysregulation of NF-κB translocation in PBMC of patients with severe sepsis and the low presence of cytoplasmic IκBα suggest that despite the successful use of drugs in animal models of sepsis, the inhibition of NF-κB activation may not be appropriate to treat septic patients. This approach may prove to be useful if it can be delivered at the onset of inflammation or within defined compartments.

SEPSIS IS ASSOCIATED WITH AN EXACERBATED PRODUCTION OF ANTI-INFLAMMATORY CYTOKINES AND MEDIATORS

Interleukin-6 (IL-6)

Involvement of IL-6 in Sepsis

Although IL-6 is often considered as an inflammatory cytokine, most of its activities are probably associated with a negative control of inflammation

thanks to its potent capacity to induce the production of acute phase proteins by the liver as well as the release of IL-lra and sTNFR [144].

Detection of IL-6 in Sepsis

The presence of IL-6 in the plasma of sepsis patients was first reported in 1989 [8, 145]. Plasma IL-6 has been observed in 64% to 100% of studied patients. Most investigators have demonstrated that levels of circulating IL-6 correlate with severity of sepsis and may predict outcome [8,9,145,146] as illustrated by the correlation between IL-6 levels and APACHE II scores [6,147]. Numerous correlations between IL-6 levels and other markers have been reported including C3a, lactate [145], circulating endotoxin [5,148], C-reactive protein (CRP) [147] and TNF [5,8,146]. IL-6 levels are similar in Gram-positive or Gram-negative sepsis [9]. Injection of endotoxin in human volunteers revealed that the peak IL-6 level was reached 2h after injection [149,150]

Interleukin-11 (IL-11)

IL-11 belongs to the IL-6 superfamily. Although IL-11 stimulated the production of several major acute phase proteins by hepatoma cells, circulating IL-11 did not significantly participate in the production of acute-phase proteins by the liver [151]. One of the major beneficial effects of IL-11 which has been described is related to its healing activity on the intestinal tract. For example, chemotherapy and radiation both damage the small intestinal mucosa barrier and lead to the entry of gastrointestinal flora into the blood. In this lethal model, IL-11 was able to protect 80% of the animals [152]. Beneficial properties of IL-11 have also been demonstrated in a rat neonatal infectious model with group B streptococci. Prophylactic use of IL-11 enhanced the survival in this model in association with an increased number of platelets [153]. Divergent reports concern IL-11 which was detected in 67% of patients with disseminated intravascular coagulation complicated by sepsis [154] but not in patients suffering from septic shock [43].

IL-1 receptor Antagonist (IL-1ra)

Involvement of IL-1ra in Sepsis

IL-lra is a natural IL-1 inhibitor. Produced by many cell types, including monocytes/macrophages, it is also produced by the liver as an acute phase protein [155]. Early treatment with IL-1ra reduced mortality from endotoxic shock [2], prevented *Staphylococcus epidermidis*-induced hypotension [156], and improved survival and hemodynamic performance in *E. coli* septic shock [157]. Depending on the dose of IL-1ra, it either reduced or enhanced lethality in a model of *Klebsiella pneumoniae* infection of new born rats [158]. In agreement with these observations, IL-1ra-deficient mice were more susceptible than controls to lethal endotoxemia [159].

Detection of IL-1ra in Sepsis

IL-1ra is present in plasma in healthy persons. Enhanced levels of IL-1ra have been regularly reported in critically ill patients, septic adults, and new born patients [6,160,161]. It may correlate with APACHE II score [6]. As an antagonist, its concentration has to be at least 100 fold higher than that of IL-1 to efficiently block the effects of this cytokine. Indeed 2,000 fold higher concentration have been noted in patients with septic shock [6]. In two patients who died within 3 h to 8 h after admission with a *Streptococcus* group A or *Neisseria meningitidis* septicemia we found a 3,400 and 61,000 fold higher concentration of IL-1ra than IL-1β, respectively [162,163]. These observations suggest that the balance between pro- and anti-inflammatory cytokines seems adequate to limit the effects of pro-inflammatory cytokines.

Soluble IL-1 Receptors (sIL-1R)

Both IL-1 receptors can be shed by the cells and bind to IL-1β. However, the soluble form of IL-1R type I has no discernable anti-inflammatory property following endotoxin administration in human volunteers [164]. This may reflect the fact that sIL-lRI has a similar affinity for IL-1α, IL-1β than for IL-1ra [165]. In contrast, the soluble form ofthe type II receptor, also known as the decoy receptor, binds IL-1β with higher affinity than IL-1ra and inhibits IL-1 activity. Plasma levels of sIL-1RII in patients with sepsis syndrome were higher than those of sIL-1RI [166].

Soluble TNF Receptors (sTNFR)

The soluble forms of the TNF receptors (sTNFRI and sTNFRII) are natural inhibitors capable of limiting TNF bioactivity. Injection into animal models of sepsis was also shown to be essentially protective [167,168]. Sepsis is associated with an enhanced plasma level of soluble TNF receptors. In children with severe meningococcemia, high levels of sTNFRI and II correlates with a poor outcome [169]. In meningococcemia as well as in sepsis, high levels of sTNFRI and II correlate with TNF-α levels [170,171]. Increased levels of sTNFR can be induced by an injection of LPS [170,171] or following injections of IL-1 [172] or TNF [173].

Interleukin-10

Involvement of IL-10 in Sepsis

IL-10 is a well known cytokine which exerts its anti-inflammatory properties particularly on monocytes/macrophages, neutrophils and T-lymphocytes. IL-10 is capable of preventing lethality in experimental endotoxemia [174] and IL-10 deficient mice were far more sensitive to LPS-induced lethality than wild-type animals [175]. In addition, neutralization of IL-10 in endotoxemia and during experimental septic peritonitis illustrated that endogenously produced IL-10 was instrumental in down-regulating the overzealous production of pro-inflammatory cytokines [176,177]. In this context, it is interesting to recall the observation by Donnelly et al. [178] that a poor prognosis in patients with ARDS was significantly associated with the lowest levels of IL-10 and IL-1ra.

Detection of IL-10 in Sepsis

Significant amounts of IL-10 are detected in the circulation of septic patients [179,180]. The highest plasma levels of this regulatory molecule are detected in the most severe cases (with shock or with poor prognosis) [178,181]. The ratio of IL-10 to TNF-α is also associated with poor outcome [181]. These observations illustrate that sepsis is not associated with a deficient anti-inflammatory response. In contrast, the exacerbated production of anti-inflammatory cytokines in sepsis cautions against a widespread use of

therapeutical approaches only targeting the pro-inflammatory mediators. Indeed, the overproduction of anti-inflammatory cytokines and mediators led to the concept of the "compensatory anti-inflammatory response syndrome" (CARS) [182]. This is further suggested by the work of P. Brantzaeg et al. [183] who showed that plasma IL-10 was in part responsible for the monocyte deactivation noticed in sepsis (see below).

Interleukin-4 and Interleukin-13

In addition to IL-10, IL-4, IL-13, transforming growth factor (TGF)-β and IFN-α also possess strong anti-inflammatory activities and a potent capacity to inhibit the synthesis of the pro-inflammatory cytokines. Each individual anti-inflammatory cytokine has been demonstrated to be capable of reducing mortality in various endotoxic or septic shock models. IL-4 prevented mortality from acute but not from chronic murine peritonitis [184]. All mice pre-treated with IL-4 survived an i.p. injection of 10^7 live *E. coli* and 10^9 *Bacteroides fragilis* which killed 90% of the control animals. Using IL-4 deficient mice it was established that IL-4 can protect against TNF-mediated cachexia and death during parasitic infection [185]. However, pre-treatment with IL-4 before the induction of sepsis was protective whereas an increased mortality was reported when IL-4 was given at the time of infection [186]. This illustrates the importance of the timing and reconfirms the idea that one should be very cautious when referring to a too simplistic dichotomy between pro- and anti-inflammatory cytokines [187].

IL-13, which shares many activities with IL-4, fully protected mice from a LD90 i.p. injection of LPS [188,189]. IL-13 blockade with anti-IL-13 antibodies significantly decreased the survival rate of mice after experimental peritonitis and enhanced tissue injury which was associated with an increased expression of many chemokines [190]. This latter result suggests that, despite the absence of detectable circulating IL-4 or IL-13 in human sepsis [69,191], these cytokines may well be involved in the control of the exacerbated release of pro-inflammatory cytokines.

Interleukin-9

IL-9 is a T-cell derived cytokine, originally described as a growth factor for T cells and mast cells. Prophylactic injections of IL-9 conferred resistance of mice challenged with a lethal concentration of *Pseudomonas aeruginosa* [192]. The protective effect was correlated with a marked decrease of serum

levels of TNF-α, IL-12 and IFN-γ as well as an increase of circulating IL-10 and IL-10 mRNA expression in the spleen. Interestingly, a shorter and lesser expression of IL-9 mRNA was observed in the spleen of mice after a lethal challenge than in mice after a sublethal bacterial challenge. To our knowledge, IL-9 has not yet been investigated in human sepsis.

Transforming Growth Factor-β (TGF-β)

Injection of TGF-β in mice before, or even together with, high doses of LPS was associated with a reduced mortality [193]. In a rat model of endotoxemia, TGF-β markedly reduced inducible NOS mRNA and protein levels in organs, arrested LPS-induced hypotension and decreased mortality [194]. Measurements of circulating TGF-β1 are controversial, most probably because of the difficulty to measure it and the fact that a latent and an active form already exist in normal plasma. Furthermore, since platelets are an important source of TGF-β1, measurements in plasma, platelet-poor plasma or sera may explain the discrepancies in the literature. Karres et al. [195] and Astiz et al [196] reported a reduced level in sera from septic patients. The mean levels of serum TGF-β1 in healthy controls were in the range of ng/ml in one study and pg/ml in the other, illustrating the difficulty linked to its measurement. On the other hand, we found enhanced levels in plasma and platelet-poor plasma in patients with sepsis [197]. We found a correlation ($r = 0.87$, $p = 0.01$) between levels of TGF-β1 in pleural effusion and in BAL fluid from septic patients whereas there was no correlation with plasma levels [198]. In a baboon septic model, Junger et al. [199] reported that active TGF-β levels increased while total TGF-β decreased. In a rat model of sepsis, circulating levels of TGF-β were found to be increased and to contribute to the depressed T-cell functions [200].

Interferon-α (IFN-α)

IFN-α prevented LPS-induced mortality in mice and reduced TNF mRNA expression in the spleen and liver [201]. The most fascinating observation was the capacity of IFN-α to be effective even when administered long after LPS. This contrasts with many reports in which the protective cytokine or drug had to be administrated before or simultaneously with LPS. Surprisingly, very few other studies have addressed the role of IFN-α in sepsis

EX VIVO CYTOKINE PRODUCTION TO MONITOR SEPSIS-ASSOCIATED IMMUNE DEPRESSION

Sepsis syndrome is associated with an exacerbated *in vivo* production of pro- and anti-inflammatory cytokines as assessed by their increased levels in the blood stream. Paradoxically, a reduced capacity of circulating leukocytes from septic patients to produce cytokines as compared to cells from healthy controls has been regularly reported. The very first observation on the hyporeactivity of circulating cells in septic patients was demonstrated with peripheral blood lymphocytes. In the initial study, Wood et al. [202] reported a decreased IL-2 production upon phytohemaglutinin (PHA) stimulation. More recently, IFN-γ production was also reported to be affected in sepsis as well as in patients with severe injury [203]. While it is often suggested that the depressed response mainly affects the production of the Th1 cytokines (IL-2, IFN-γ), we demonstrated that the production of Th2 cytokines (IL-5, IL-10) could also be altered and that the nature of the triggering agent itself influences the observation [204]. Monocyte reactivity to LPS stimulation has been particularly studied in isolated monocytes and in whole-blood assays. Monocytes from septic patients had a diminished capacity to release TNF-α, IL-1α, IL-1β, IL-6, IL-10 and IL-12 [203,205,206,207,208] whereas this was not the case for IL-1ra [206]. Reduced cytokine production has also been observed with other stimuli such as silica, staphylococcal enterotoxin B, killed *Streptococcus* and *Staphylococcus* [196,203,209,210]. Similar hyporeactivity has been reported for the production of IL-1β, IL-1ra and IL-8 by LPS-activated neutrophils from septic patients [211,212,213].

Although the anergy of the cells observed in septic patients has been associated with endotoxin tolerance [211], this phenomenon is neither specific for endotoxin [214] nor for septic patients. Indeed, in many stressful conditions including trauma, thermal injury, hemorrhage, and severe surgery, hyporesponsiveness of circulating leukocytes and low cytokine production have been regularly reported and associated with immune depression observed in these patients.

Thus, during systemic inflammation, systemic inflammatory response syndrome (SIRS) and CARS seem to be present simultaneously; SIRS predominating within the inflamed tissues while in the blood, leukocytes show hyporeactivity (Figure 3).

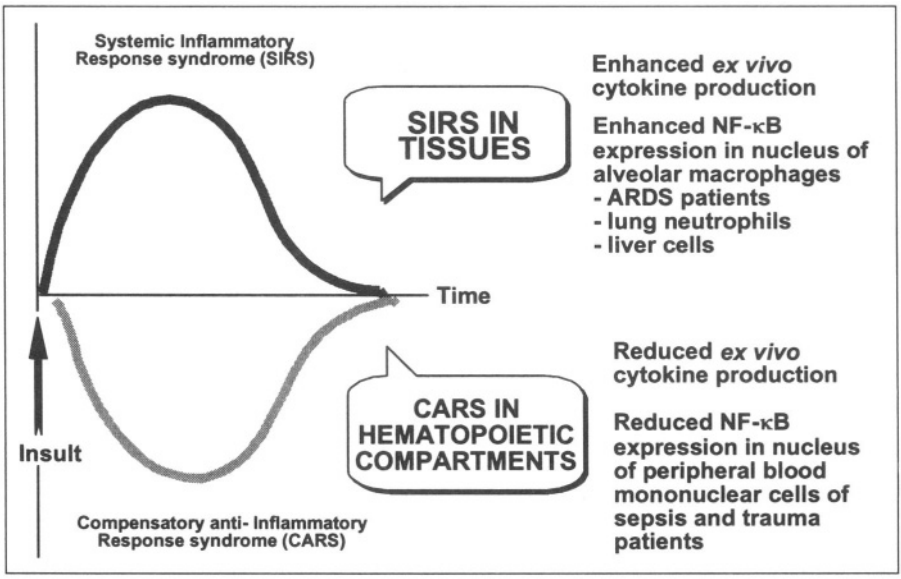

***Figure 3**. Compartmentalization of the inflammatory response.*

WHICH CYTOKINE MEASUREMENTS ?

We will not discuss in detail the technical aspects linked to cytokine measurements as these have been addressed elsewhere [215]. We will just discuss the fact that cytokines assessed in any biological fluids represent the tip of the iceberg [216]. Indeed, once the specific mRNA has been translated, cytokines can be found within the cellular compartments associated with protein synthesis, and some cytokines such as **IL-1α, TNF-α,** IL-10, **IFN-γ,** and IL-15 can be found as a constitutive compound of the cell membrane. Present in the cellular environment, cytokines can be trapped by surrounding cells which possess specific receptors; finally, once bound to the receptors, cytokines are usually internalized within the cells (Figure 4). Thus, we showed that **IL-1α, IL-1β** and **TNF-α** could be found associated to monocytes of septic patients [9]. Interestingly, at the end of patient follow up, while most survivors did not have any more detectable circulating TNF, a majority still had detectable cell-associated TNF; recent studies suggest that this could be a membrane form of TNF. Indeed in patients with systemic injuries, enhanced expression of membrane TNF was reported whereas no intracellular TNF could be detected [217]. Similarly an increased expression of functionally active membrane-associated TNF has also been demonstrated

on alveolar macrophages from patients with ARDS [218]. Flow cytometry analysis confirmed the presence of IL-1β positive cells among circulating leukocytes of intensive care unit patients [219]. More recently we investigated cell-associated IL-8, and found that tremendous amounts of IL-8 could be found associated to circulating neutrophils and mononuclear cells [47]. Lower, but significant amounts of IL-8 were also associated with red blood cells via their Duffy antigen. In ARDS patients, the identification of numerous IL-8 positive alveolar macrophages by immunocytochemistry has confirmed the putative detrimental role of IL-8 in the development of that syndrome [220].

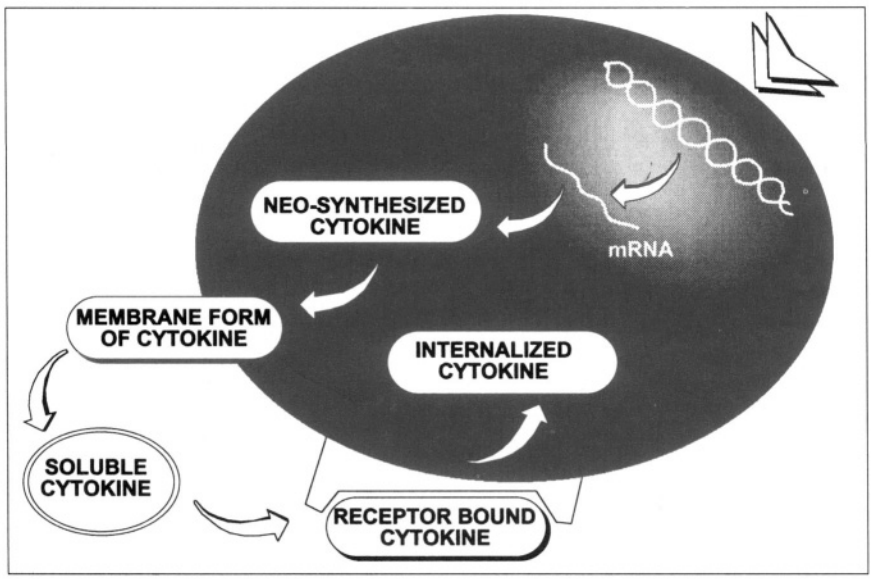

***Figure 4**. The source of cell-associated cytokines (adapted from [215])*

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